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This work describes the development of a novel system for efficient and rapid introduction of genes into specific tissues or cells in mice. Detailed analysis of gene function often relies upon introduction of genes into an animal model system. We are developing a retroviral-based system in which controlled expression of the cellular receptor for the virus in a defined pattern determines the site(s) of infection. Using this retroviral gene delivery system, a gene can be introduced not only in a spatial-specific pattern but also in a temporally-controlled manner using a retroviral vector. For these studies we expressed the receptor for the avian virus Rous sarcoma virus in mammary cells in transgenic mice using a MMTV LTR. The ability to selectively target retroviral infection has numerous applications in areas as diverse as developmental biology, gene therapy, and oncogenesis. Here we will attempt to utilize this system for targeted expression of genes in mammary cells to rapidly assess the potential of these genes to induce mammary tumors in an animal model.

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FOREWORD

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✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

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PI - Signature 2/13/88 Date

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INTRODUCTION

Presently, the most common way to analyze gene function in a particular cell type *in vivo* is to generate a new transgenic line for each gene under study - a costly and time consuming endeavor. Here we describe an approach which utilizes mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor) to target infection of retroviral vectors *in vivo.* This approach allows directed infection, and thus directed gene expression, of cells expressing the viral receptor and may provide a rapid and efficient method to test the mammary tumorigenic potential of genes in an animal model. An important difference between this approach and the more traditional method of testing gene function in transgenic mice is that infection, and thus gene expression, can be temporally controlled. This allows assessment of differences in oncogenic potential of genes at different stages of mammary gland development. In addition, by allowing the gene to be introduced after gland development has occurred, it avoids effect of the genes on early gland development and might therefore provide a more relevant model of tumorigenesis. Finally, it should be possible to introduce multiple oncogenes by co-infection thereby allowing questions of synergy between these genes to be addressed.

The goals of this research project as defined by the Statement of Work are:

TASK 1, Generate transgenic mice expressing RSV receptor in specific tissues.
- a. construct MMTV LTR promoter-RSV receptor transgene.
- b. test expression of MMTV transgene in C57 mammary epithelial cells in culture. Test for susceptibility to RSV vectors.
- c. produce transgenic mice and establish lines with MMTV LTR-RSV receptor transgene

TASK 2, Characterize expression of transgene in mammary gland and other tissues.
- a. RNase protection analysis of receptor transcripts in tissues of transgenics
- b. Western blot analysis of receptor protein expression in tissues of transgenics

TASK 3, Infect transgenic animals with RSV vectors.
- a. develop protocol for infection which gives maximal infection of mammary cells (test route of infection, timing with respect to age, pregnancy etc.)
- b. produce RSV vectors carrying histochemical marker genes
- c. infect mice with vectors carrying markers (alkaline phosphatase and beta galactosidase). Score for infection by histochemical staining.

d. infect mice with int-1 and v-myc RSV vectors. analyze tumors.

In preparation for the project outlined above, we collaborated with Steve Hughes at NCI to produced mice carrying RSV receptor transgenes. Using a muscle specific alpha-actin promoter/tva construct, we established five mouse lines carrying this transgene. Characterization of these lines by Western blot analysis demonstrated that the receptor was specifically expressed in several types of muscle. Although the level and pattern of expression in muscle vary for each of the transgenic lines, these experiments demonstrate that in general, the receptor can efficiently expressed without deleterious effects. Furthermore, using mice carrying a ß-actin promoter/tva construct Dr. Hughes lab has demonstrated expression of Tva in numerous cell types including early embryonic cells. Together the results with these two different promoters suggested that expression of Tva in many different tissues and at numerous developmental periods was not detrimental. Therefore, it we felt it was reasonable to attempt making transgenic animals expressing Tva in the mammary gland.

In published experiments using the α-actin-tva transgenic mice we directly demonstrated targeted retroviral infection *in vivo* . 2000-5000 infectious units of an RSV vector carrying the bacterial alkaline phosphatase gene (RCAS(A)-BAP) were injected into the thigh muscle of 5 day old mice. At d5 post-birth there is significant myogenesis occurring such that the myoblasts, if susceptible to RSV-A, should be good targets for infection. Controls for the experiments included injection of a subgroup E RCAS-BAP vector which should not utilize the subgroup A receptor and injection of non transgenic littermates. Infection was seen only when the subgroup A virus was injected into transgenic mice. Several hundred infected myoblasts or myotubes are spread throughout the muscle and infection did not seem to be localized at the injection site. Furthermore, by injecting avian cells expressing the RSV vectors rather than the virus stock, infection of the myoblasts was dramatically increased such that thousands of cells appear to be infected. These experiments provide proof of principal for the use of Tva to efficiently target cells for RSV-A infection *in vivo.*

BODY

This year's report concentrates on work developing viral vectors for introduction of foreign genes into mammary epithelial cells in vivo. In addition, this report details ongoing research aimed at defining optimal parameters for transfer into the mammary epithelial cells in the gland. The report is divided into sections based on the tasks described in the Statement of Work.

Task 1 Transgenic mice

Last year we reported the successful establishment of two mouse lines that carried the MMTV-tva transgene. In last year's report, I alluded to problems with mouse hepatitis virus (MHV) infection of our transgenic lines that were hampering our production of transgenics for these lines. Because of MHV we were required to move these animals to a different ("dirtier") facility, while we prepared to re-derive the lines by embryo transfer. Unfortunately, at this facility losses due to the MHV infection coupled with poor breeding of our C57BL-6 backcrossed transgenic animals (possibly due to a concurrent pinworm infection) caused both lines #1 and #22 to be lost. Thus, our work this year is to re-establish these mice. While we were having these problems, Harold Varmus (NIH) informed us that his lab is also interested in establishing MMTV-tva mice. Based on our work that was reported last year, the Varmus lab is confident that they will establish MMTV-tva lines. While we are also working to produce new MMTV-tva transgenics using the same transgene construct we employed previously, we also recognize that the efforts of the Varmus lab will duplicate ours. Therefore, my lab has concentrated on experiments described below for developing vectors that will allow efficient introduction of genes into the transgenic mice.

In addition, I should point out that several labs are now using our system to target various tissues in the mouse by attaching *tva* to different tissue-specific promoters. In all cases thus far, *tva* can be expressed in the appropriate target tissue. Furthermore, in at least one setting, these transgenic animals have been successfully used to analyze tumor induction (H. Varmus and E. Holland, personal communication).

Task 2 Characterization of transgene expression

Because of the problems breeding the transgenic mice, no animals were available for analysis of transgene expression. Work on this task must be deferred until the transgenic lines are re-established.

Task 3 *in vivo* infection of mammary epithelial cells

A. Additional infection studies in transgenic mice.

Last year we reported preliminary experiments suggesting that the MMTV-tva mice had been infected with an MLV(RSV) virus that carried a β-galactosidase reporter gene. Further analysis of additional infected mice infected with MLV(EnvA) β-gal viruses suggests that this conclusion may have been premature. In the studies reported last year pregnant mice were injected with MLV(EnvA) β-gal. Three weeks after injection the glands were harvested and analyzed for betagalactosidase expression by staining of whole mounts. In repeating this work, we found that while there is significant β-gal activity detected in the infected glands, glands from uninfected animals also display significant staining, suggesting

that during lactation there is a high level of endogenous β-gal activity in the mammary gland (Figure 1). To confirm this, we injected virus into one #4 and one #3 gland in a pregnant MMTV-tva transgenic mouse, waited three weeks, then harvested and stained all 4 #3 and #4 glands. When infected and uninfected glands from the same animal were compared, there was no difference in the level of staining seen (data not shown), confirming that the results suggesting infection based on β-gal staining are likely in error. As described below we are currently evaluating if other marker genes will prove more useful in the mammary gland than β-gal.

B. Virus vectors and stocks

Last year we described the use of a transient system to produce MLV viruses carrying the RSV EnvA protein which would allow directed infection of these viruses into cells expressing tva. One advantage of this system over replication competent RSV vectors is that the simpler defective MLV vector backbone allows easier insertion of genes and accommodation of larger inserts. In addition, the RSV vector encodes the structural proteins of this virus and when introduced into mice after the neonatal stage is likely to elicit an immune response, potentially resulting in clearance of infected cells carrying the transduced gene. The defective MLV vectors do not encode any structural genes. To fully take advantage of the transient MLV system we needed to extensively modify the original vector described for use in this system (pHIT110). The original vector was quite large (9.1kb) and contained a G418 resistance gene which we wanted to delete to avoid potential immune recognition of infected cells. The plasmid we constructed, pHIT110 poly, is much smaller (5.1kb), is derived entirely from known sequences, and contains a polylinker region with ten unique sites for introducing genes of interest (Figure 2).

Another feature required for vectors injected into the gland is high titer. This requirement stems from the fact that very small volumes can be injected (maximum of 50μl). To achieve the highest titers possible we would like to concentrate the viruses. We have experimented with two procedures to produce high titer stocks, ultracentrifugation and ultrafiltration. Through a series of experiments, we find that a high level of concentration can be achieved for EnvA pseudotyped viruses by ultracentrifugation (15 minutes, 80K X g) while ultrafiltration (Pall, MacroSep 100Kd cutoff) results in a significant loss of infectivity. It appears that the most critical parameter for successful concentration by ultracentrifugation is slow resuspension of the pelleted virions (overnight at 4° without vortexing works best). Using two sequential concentration steps we have achieved a greater than 200-fold increase in MLV(EnvA) β-gal titers (final titer $2X10^7$ IU/ml).

To avoid potential problems associated with endogenous galactosidase activity discussed above, we are constructing vectors that carry other markers for analysis of in vivo

infections. One such vector is an MLV genome encoding green fluorescent protein (GFP) or variants of GFP that have been optimized for expression in eukaryotic cells or that have shifted excitation and emission spectra compared to wild type GFP. To avoid potential problems of in vivo immune responses to other proteins encoded by the MLV vectors, we are using the minimal viral vector described above that encodes GFP (pHIT-GFP) but no selectable marker gene. In addition, we have obtained a vector encoding alkaline phosphatase from Dusty Miller (pLNCAP) which encodes G418 resistance as well as alkaline phosphatase. While the pLNCAP vector will be used in preliminary infection studies optimizing infection parameters (see below), we are currently moving the AP gene into pHIT110poly to produce pHIT-AP which should avoid immune response problems mentioned above and from our experience should result in higher titer MLV(EnvA) viruses.

C. Optimizing in vivo infection protocol.

We are utilizing MLV vectors carrying envelope proteins that are known to direct infection of a wide variety of cells in culture in an attempt to define optimal parameters for retroviral infection of mammary epithelial cells. This work is in preparation for studies on directed infection with MLV(EnvA) viruses when the MMTV-tva transgenic animals become available.

Toward this end, we have begun producing MLV viruses pseudotyped with either vesicular stomatitis virus glycoprotein (VSV-G), influenza hemagglutinin (HA), or the Ebola Zaire strain glycoprotein (EboGP). Both these envelope proteins mediate infection of a wide variety of cell types in numerous species, thus it is highly likely that they will allow infection of mammary epithelial cells. We chose to use these envelopes instead of MLV ecotropic envelope (which will also mediate infection of murine cells) because of the stability of VSV-G, HA, and EboGP. In all three cases viral stocks can be concentrated by centrifugation (Table 1) to produce the titers required for in vivo administration of viruses. This is not the case for the MLV ecotropic envelope protein which is more fragile and does not allow concentration.

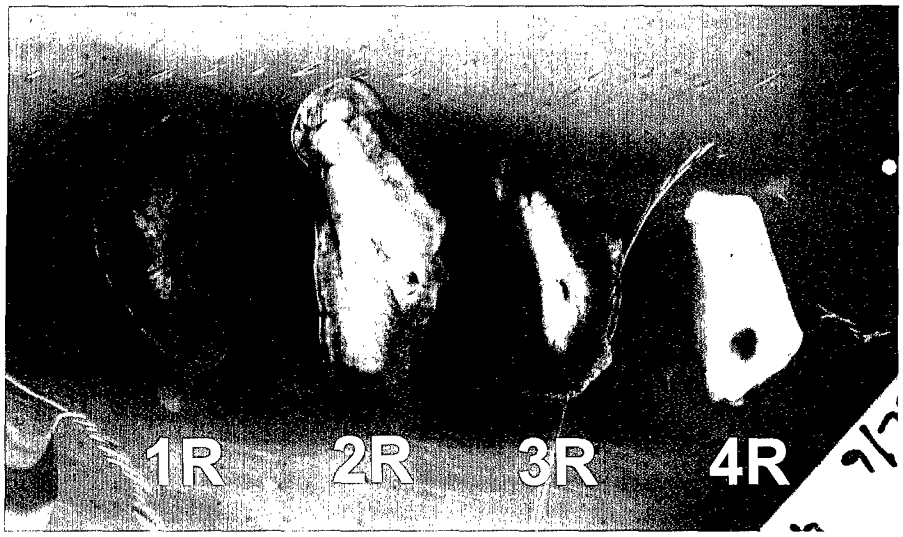
MLV(EboGP), MLV(HA), and MLV(VSV-G) viruses carrying pMX-GFP or pLNCAP are currently being prepared and concentrated. They will be injected directly into the mammary gland of female mice at different stages of puberty or pregnancy to determine conditions during which mammary epithelial cells are good targets for retroviral infection in vivo. The optimal conditions determined by these studies will then be employed in future studies with MLV(EnvA) vectors and MMTV-tva mice.

CONCLUSIONS

The overall goal of this project is to develop a good animal model in which genes could be rapidly and efficiently introduced into mammary epithelial cells in vivo. This year we have encountered significant problems with our transgenic animals. Therefore, we have concentrated on developing vectors that will be used when the transgenic mice are available. Analysis of additional animals in which mammary gland infection was attempted using vectors expressing a β-gal marker gene demonstrates that there is significant endogenous β-gal activity in the gland that may make use of this marker gene problematic. In experiments with glands from mice that are not lactating we only see significant endogenous β-gal activity in the lymph node that underlies the mammary gland. To circumvent this detection problem we are developing vectors expressing alkaline phosphatase or GFP.

To prepare for the studies on the MMTV-tva transgenics, we are using retroviral vectors with broad tropism, conferred by the VSV, influenza, and Ebola glycoproteins, to analyze conditions that favor retroviral infection. These studies can be conducted in non-transgenic animals. Because these envelope proteins are capable of directing infection of a wide variety of cells and because we will inject the virus stocks directly into the mammary gland at times when epithelial cell proliferation is known to occur, then it seems likely that we will transduce primarily epithelial cells with these vectors. If this is the case (and we should be able to distinguish on sections if the epithelial cells are infected), then it is likely these pilot experiments will define conditions which result in the most efficient infection of mammary epithelial cells in vivo.

A modified MLV vector, pHIT110 poly, has also been constructed. This vector deletes all protein encoding sequences to avoid any potential problems of immune response to the selectable markers. pHIT poly retains a chimeric 5' LTR that utilizes the CMV promoter enhancer for high level expression of the MLV genomic RNA and has an extended packaging sequence for efficient incorporation during transient production of virus. Experiments with a derivative of pHI110 poly encoding β-gal demonstrated that this vector works well for production of high titer stocks. Therefore, pHIT110 poly is being employed for all the viral constructs which will be used in MMTV-tva mice.



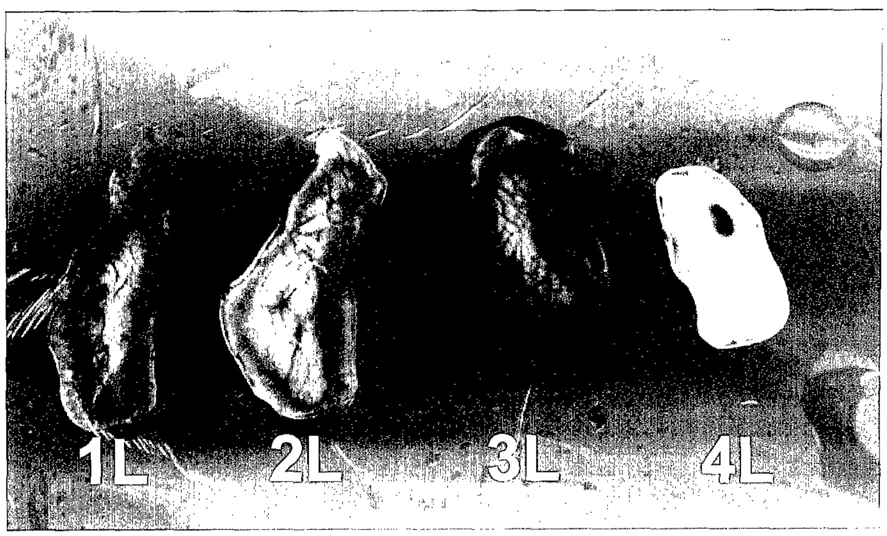


Figure 1. Betagalactosidase activity in lactating mammary glands. The right #4 gland of three mice was injected at approximately day 17 of pregnancy with either cell free MLV(EnvA) β-gal virus (3R), 293T cells producing this virus (1R) or mock injected (2R). The left gland was kept as an uninfected control (1L, 2L, 3L). Three weeks later the mice were euthanized and the glands mounted on slides and stained as whole mounts for β-gal activity. Both right and left glands were harvested from an uninjected, non-lactating 10 week old mouse (4L and 4R). As can been seen, the level of β-gal staining is independent of the injected virus and seems to be specific for the state of the gland in each mouse.

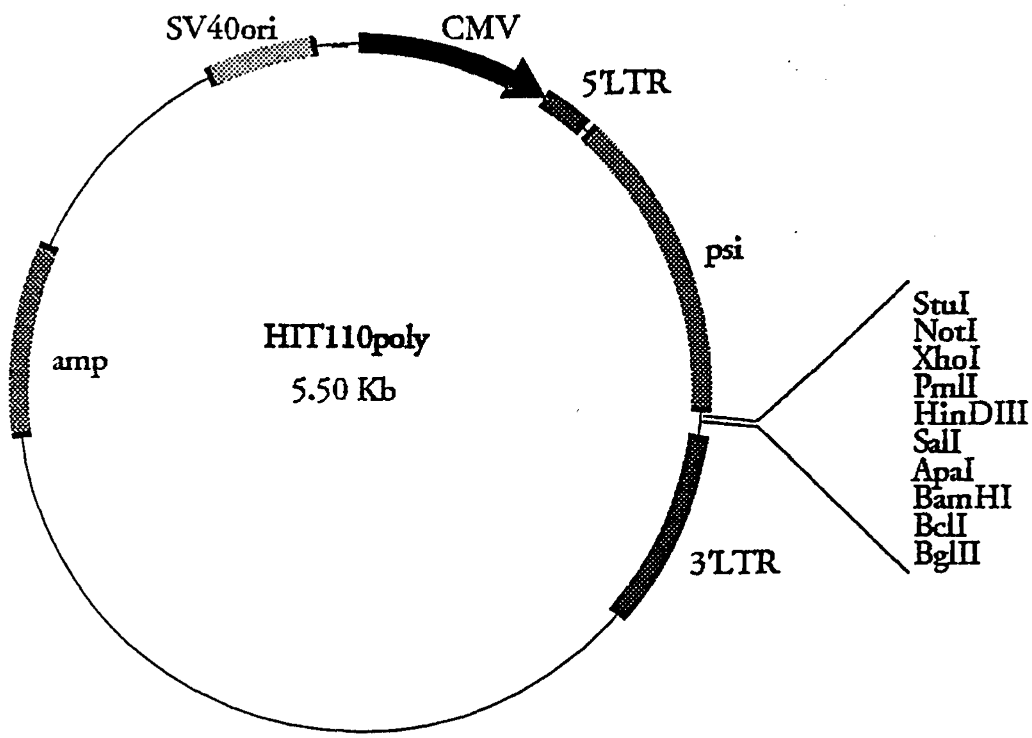


Figure 2. Map of pHIT110 poly MLV vector. A modified vector for transient production of viruses from 293T cells was constructed. The chimeric CMV promoter-enhancer-5'LTR from pHIT110 was cloned into the plasmid vector pSP72 (Promega), then the MSV 3' LTR and polylinker sequences from pSL1180 (Pharmacia) were inserted to produce pHIT110 poly.